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Genetics

Stimulation of homologous recombination in plants by expression of the bacterial resolvase RuvC

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ABSTRACT Targeted gene disruption exploits homologous recombination (HR) as a powerful reverse genetic tool, for example, in bacteria, yeast, and transgenic knockout mice, but it has not been applied to plants, owing to the low frequency of HR and the lack of recombinogenic mutants. To increase the frequency of HR in plants, we constructed transgenic tobacco lines carrying the *Escherichia coli* RuvC gene fused to a plant viral nuclear localization signal. We show that RuvC, encoding an endonuclease that binds to and resolves recombination intermediates (Holliday junctions) is properly transcribed in these lines and stimulates HR. We observed a 12-fold stimulation of somatic crossover between genomic sequences, a 11-fold stimulation of intrachromosomal recombination, and a 56-fold increase for the frequency of extrachromosomal recombination between plasmids co-transformed into young leaves via particle bombardment. This stimulating effect may be transferred to any plant species to obtain recombinogenic plants and thus constitutes an important step toward gene targeting.

Gene targeting (GT) involves the disruption or replacement of an endogenous allele by one manipulated *in vitro*. This replacement requires that after transfection into the target cell the transgene recombines with the target allele by homologous recombination (HR) by virtue of sharing extensive sequence similarity, rather than integrating randomly by illegitimate recombination. As HR occurs efficiently in many lower eukaryotes such as budding yeast and in bacteria, GT has proved to be a very powerful reverse genetic tool to study gene function. The GT technology is also now routine, if not still laborious in the generation of transgenic knockout mice, and has provided models for human genetic disease and the role of homeotic genes in development (1).

The mechanism of HR has long been an elusive, yet fascinating, problem. Studies in prokaryotes and lower eukaryotes have provided much insight into the nature of this process, the recombination intermediates, the genes, and the proteins involved (2–4). The basic steps of HR are listed below together with the proteins required in *Escherichia coli*: (i) initiation of HR by a DNA double-strand break and/or single-strand DNA formation by the RecBCD complex; (ii) exchange of DNA strands, including homology recognition and strand displacement, done by RecA-like proteins; (iii) heteroduplex extension, with branch or bubble migration, performed by RuvA plus RuvB or RecG to yield a recombination intermediate, a four-way DNA junction named the Holliday junction; and (iv) resolution of this heteroduplex Holliday junction by the endonuclease RuvC. Steps iii and iv have only very recently been elucidated: in *E. coli* the RuvA and RuvB proteins (encoded on one operon) have been shown to form a complex promoting ATP-dependent branch migra-

tion of Holliday junctions, a process of high importance for the formation of heteroduplex DNA. A second operon encodes RuvC and the *orf-26* gene; RuvC is the endonuclease that binds specifically, as a dimer, to Holliday junctions and promotes a subsequent resolution of Holliday junctions. Mutations in RuvA, RuvB, or RuvC result in defects in recombination and DNA repair. Genetic and biochemical studies indicate that branch migration and resolution are coupled by direct interactions between these three Ruv proteins, possibly by the formation of a RuvABC complex (4). A RuvC homolog, CCE1 (cruciform cutting endonuclease), has been reported for mitochondria of *Saccharomyces cerevisiae* (5) and for the fission yeast *Schizosaccharomyces pombe* (6). No plant homolog has yet been found.

In plants, the frequency of HR is low, as evidenced in the Kb/centiMorgan ratio, which is typically much higher than in lower eukaryotes or prokaryotes (7). However, GT has been demonstrated after direct delivery of DNA (8, 9) or by *Agrobacterium*-mediated infection (10, 11) using engineered genomic targets, albeit at a frequency 10^{-4} to 10^{-5} -fold lower than illegitimate integration. There are two recent reports of GT in *Arabidopsis* via *Agrobacterium* infiltration in planta (12, 13). Unfortunately, the number of events was so small that it is not possible to obtain good GT frequency estimates. Low frequencies of HR also have been reported when quantification was done for intrachromosomal recombination (ICR) in tobacco and *Arabidopsis* (14–16). Similarly, the rate of somatic crossover between homologous chromosomes was shown to be very low (17, 18). Although low frequencies of HR are probably important to maintain stability of the repetitive plant genome, they are nonetheless a major hindrance to the implementation of the powerful GT technology in plants.

Why HR is inefficient in plants remains obscure. The recent isolation of *Arabidopsis* mutants affected in somatic and/or meiotic HR (19) might help identify the components of the plant recombination machinery and reveal how HR is regulated in plants. An alternative approach to overcoming the problem of low rates of HR is to overexpress well-characterized HR-related genes from heterologous species in plants and test how this affects HR in plants. By using this approach, (20) it was shown that the overexpression of the bacterial *RecA* gene in plants is associated with a 10-fold increase in the rate of intrachromosomal HR, suggesting that expression of the plant *RecA* homologs partially limits the rates of HR. Additional components must be tested to obtain a more comprehensive understanding of the control of HR in plants.

This paper was submitted directly (Track 11) to the *Proceedings* office. Abbreviations: HR, homologous recombination; GT, gene targeting; ICR, intrachromosomal recombination; GUS, β -glucuronidase; W1, wild type; NLS, nuclear localization signal; ECR, extrachromosomal recombination.

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Here, we constructed transgenic tobacco plants expressing *RuvC* and show that it is associated with a 12-fold increase in somatic crossover between genomic sequences, with an 11-fold increase in ICR, and with a 56-fold increase in extrachromosomal recombination (ECR) between plasmids cotransformed into leaves via particle bombardment. We discuss possible mechanisms leading to this increase as well as the possible applications for GT improvement.

MATERIALS AND METHODS

We built a series of constructs for studying the effect of *RuvC* on HR in tobacco plants. Plasmid pJD330, kindly provided by V. Walbot (Stanford University, Stanford CA), is a Bluescript derivative carrying the β -glucuronidase (*GUS*) reporter gene driven by the cauliflower mosaic virus 35S promoter fused to the tobacco mosaic virus translational enhancer Ω (21). Recombination partners were constructed, each with a different deletion in the *GUS* gene as described (22): one partner, 3' Δ *GUS*, (pGS001) carries a 700-bp (*SspI-EcoRI*) deletion in the 3' region of *GUS*. The second partner, 5' Δ *GUS* (pGS003) carries a 12-bp 5' deletion including the ATG initiation codon of *GUS*. Neither plasmid partner alone showed any *GUS* activity in transient assays. The *RuvC* sequence was obtained by PCR using two primers CS' and C3': 5'-GTCGACCATGGCTATTATTCCTCGGC-3', 5'-GCATGCTAAGATCTACGCAGTCGCCCTCTCGC-3', which carry *SphI* and *BglII* sites, respectively for use in further cloning. After amplification, a 520-bp amplified fragment was isolated, cloned, and subsequently fully sequenced to confirm that no amplification errors occurred. A *Sall-SphI* fragment of the *RuvC* gene was subcloned into identical sites of the pCd vector (a Bluescript derivative vector), giving rise to plasmid pGS021, which contains the *RuvC* ORF under the control of the 35S promoter and with termination sequences of *Octopine synthase* (*ocs*). A 230-bp *SphI-BglII* fragment carrying a nuclear localization signal (NLS) (kindly given to us by Vitaly Citovsky, State University of New York, Stony Brook) allowing protein targeting to the nucleus and taken from the *Nla* gene of tobacco etch virus (23) was subcloned into the same sites of plasmid pGS021, giving rise to plasmid pGS022, consisting of the 35S-*RuvC*-NLS-*ocs* 3' components. A 2,050-bp *Asp718-SpeI* fragment including the four components was subcloned into the *Asp718-XbaI* sites of the pZP111 binary vector (24), giving rise to plasmid pGS023.

Tobacco Transformation. The binary vector mentioned above was introduced by electroporation into the *Agrobacterium tumefaciens* LB4404 strain (25). The transformed *Agrobacterium* strains were used to infect leaf discs of two *Nicotiana tabacum* cultivars (cv. Samsun-NN and cv. Xanthi) and regenerate transgenic plants as described by Horsch (26). All of the plants [wild type (WT) and transgenic] in the Xanthi background were heterozygote for the *Sulfur* chlorophyll mutation (*Su/su*). All transgenic plants carried transformation markers that confer resistance to kanamycin (100 mg/liter). The number of T-DNA copies was determined on the basis of kanamycin resistance frequency in T₂ self-pollinated seedlings and by Southern blot analysis (data not shown).

For the ICR assay, transgenic tobacco plants transformed with a construct that enables us to monitor HR through reactivation of the *GUS* gene were used. These plants were kindly provided by Barbara Hohn (Friedrich Miescher Institute, Basel, Switzerland) and are described by Puchta *et al.* (16). *GUS* activity was determined by histochemical staining of 3-week-old seedlings as described (22).

Biolistic Transformation and the ECR Assay. The 3' Δ *GUS* plasmid (pGS001), with and without the 5' Δ *GUS* (pGS003) plasmid linearized at its unique *SpeI* restriction site, was transformed into leaves of WT and *RuvC*-expressing plants through biolistic bombardment, using the helium-driven PDS-

1000/He system (Bio-Rad), according to the manufacturer's instructions. Seven leaves, 3–4 cm long taken from WT Samsun-NN or from *RuvC*-expressing plants in the same Samsun-NN background, were biolistically transformed, and after 30 hr of recovery, *GUS* activity was determined by histochemical staining as described (22). Monitoring of HR was done by counting the number of blue spots per bombarded leaf, as seen under a binocular microscope.

RNA Extraction and Northern Blotting. Plant RNA samples were isolated from young leaves of WT Xanthi plant and transgenic lines expressing *RuvC* in the Xanthi background with the Tri Reagent-RNA/DNA/protein isolation reagent kit (Molecular Research Center, Cincinnati). Northern blot analysis was performed with a nylon membrane following the manufacturer's instructions.

RESULTS

Expression of *RuvC* in Plants. To overproduce the bacterial *RuvC* protein in plant nuclei, an expression vector carrying the *RuvC* ORF downstream of the 35S promoter with a translational fusion to the carboxyl terminus of a NLS was cloned in a T-DNA binary vector (Fig. 1A). This vector was transformed into tobacco plants by agroinfection, and expression of *RuvC* was tested by Northern blot analysis in T1 plants and WT. As shown in Fig. 1B, an 800-nt fragment was detected from five independent transformation events. The size of this fragment is, as expected for the pGS022 construct, suggesting that *RuvC* mRNA is properly processed, and the band intensity (out of the total RNA) suggests that *RuvC* transcript is relatively abundant in the transgenic plants. These transgenic lines did not show any indication of retarded growth, suggesting that the transformation procedure and the expression of the *RuvC*-NLS transcript did not interfere with the normal pattern of plant development. Similarly, there was no decrease in pollen fertility, and cytogenetic analyses performed at different stages of meiosis did not provide evidence for chromosomal loss or breakage (data not shown). This finding suggests that overall these plants are relatively genetically stable.

Increased ECR in *RuvC*-Expressing Plants. A rapid assay to test the recombinogenicity of *RuvC*-expressing plants is to quantify the frequency of homologous recombination between extrachromosomal molecules: two plasmids, pGS001 and pGS003, were cotransformed, via particle bombardment, into leaves of transgenic plants expressing *RuvC* and WT control plants. *RuvC*-expressing plants were kanamycin-resistant progeny of T1 plants and therefore segregate for *RuvC* homozygous and heterozygous plants. Both WT and transgenic plants are of the same Samsun-NN genetic background. Each of these plasmids contains a different mutation that prevents *GUS* expression (Fig. 2A): pGS001 has a deletion in the 3' end (3' Δ *GUS* in Fig. 2), and pGS003, a deletion in the 5' end (5' Δ *GUS* in Fig. 2). The two plasmids share 1,800 bp of identical sequences, and *GUS* activity can be restored upon HR between the two plasmids and can be quantified by the number of blue spots (Fig. 2). Linearization of pGS003 at the *SpeI* site, located between the 35S promoter and the *GUS* gene (Fig. 2), was done to increase the efficiency of HR in the assay, in accordance with findings of various labs (27). This assay is similar in principle to the previously described ECR bombardment assay (28) with different recombination substrate molecules. Both the 3' and the 5' deleted *GUS* plasmids were found to be *GUS* negative when transformed separately either in WT tobacco or *RuvC*-expressing plants (Fig. 2A). However, after cotransformation of the two plasmids, blue spots were detected in leaves of *RuvC*-expressing plants and leaves of the WT line (Fig. 2C and D). Blue spots were counted for each bombarded leaf under a binocular microscope. An average of two spots per leaf was detected in the WT (Fig. 2C), whereas an average of 111 spots was detected in leaves of the *RuvC*-

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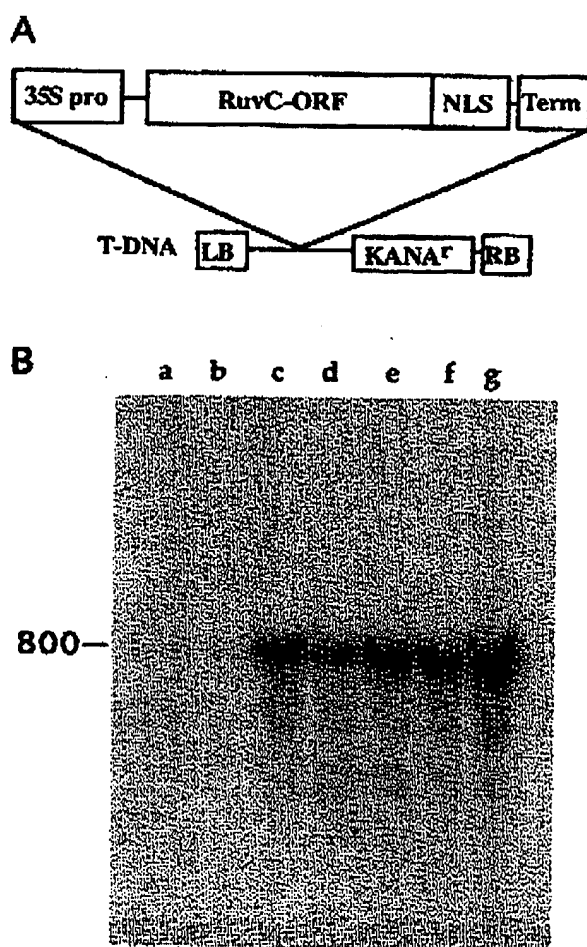


FIG. 1. Expression of RuvC in transgenic plants. (A) The expression vector contains the RuvC ORF translationally fused in the carboxyl-terminal region to the NLS derived from the tobacco etch virus (23), and cloned between the cauliflower 35S promoter (35S pro) and the transcription termination region from the octopine synthase gene (Term). This expression cassette was cloned into the pZP111 binary vector (24), giving rise to clone pGS023, and was transformed in tobacco. (B) Transcription of RuvC was determined by Northern blotting of total RNA and hybridization with the RuvC ORF fragment as a probe. An 800-nt transcript was detected in five independent transgenic plants (background of *Su/su* cv. Xanthi) transformed with pGS023 (lanes c–g), but not in the nontransformed control *Su/su* Xanthi line (lanes a and b).

expressing plants treated under the same transformation conditions (Fig. 2D), i.e., *RuvC* expression was associated with a ~56-fold increase in ECR. The frequency of blue spots observed with a positive 35S-GUS control was similar in *RuvC*-expressing plants or in the WT control (Fig. 2B), indicating that *RuvC* does not improve transformability or expression of the bombarded leaves (data not shown). The blue spots were bigger when bombardment was done with 35S-GUS than with the recombination partners (Fig. 2B vs. D), presumably because of the larger copy number of GUS-expressing plasmid molecules transformed with 35S (all the molecules on the bead) than with the cotransformed deleted recombination substrates (only recombinant molecules).

Increased ICR in *RuvC*-Expressing Plants. The effect of *RuvC* on ICR was determined by using the previously described ICR "GU-US" assay. According to this assay, ICR

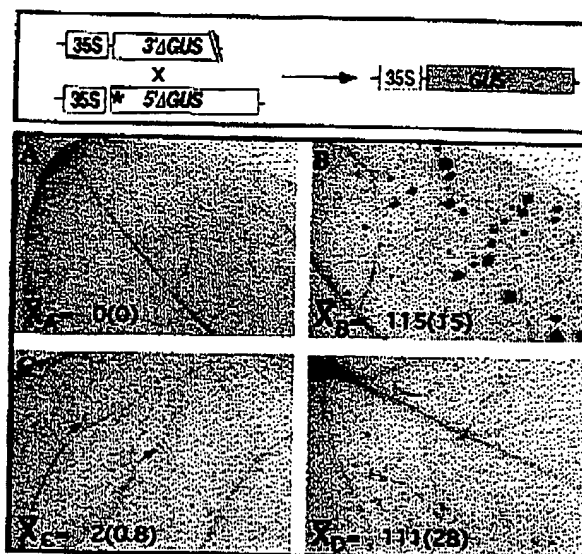


FIG. 2. ECR in *RuvC*-expressing plants. (Top) A schematic representation of the constructs used for the ECR assay is shown. Homologous recombination between two defective GUS genes, one of which is deleted in the 3' region (3'ΔGUS) and one in the 5' region (5'ΔGUS), gives rise to a GUS active gene (the blue box on the Top Right and the blue spots in the Middle and Bottom). (Middle and Bottom) Pictures of tobacco leaves (cv. Samsun-NN) that underwent bombardment and staining for GUS activity are shown, together with the average number of blue spots per leaf and the SEM in parenthesis. (A–C) Bombardment of the 3'ΔGUS or of the 5'ΔGUS plasmid alone (A) and of the 35S-GUS gene (B), and cotransformation of the 3'ΔGUS and 5'ΔGUS-containing plasmids into WT tobacco (C). (D) Cotransformation of the 3'ΔGUS and 5'ΔGUS plasmids in transgenic plants expressing *RuvC*.

events are recognized as blue sectors after histochemical staining of seedlings. It was shown that these sectors are obtained through the reactivation of a GUS reporter gene via HR between two directly repeated truncated GUS fragments (GU and US), which share a 0.6-kb long overlap (14). We have compared the occurrence of blue sectors in the cotyledons and in the first and second true leaves of F_1 (*RuvC* X GU-US) seedlings of a cross between *RuvC*-expressing plants [in the *N. tabacum* cv Samsun (N/N) background] and plants homozygous for the GU-US construct, versus the F_1 (Samsun X GU-US) seedlings of a cross between the *N. tabacum* cv Samsun (N/N) and plants homozygous for the GU-US construct. In both crosses the GU-US construct is in a hemizygote dose, and the genetic background is similar. Therefore, the differences in frequency of blue sectors between the two crosses, as shown in Table 1, are most probably related to the activity of *RuvC*. The expression of *RuvC* was associated with

Table 1. Frequency of blue sectors in seedlings of plants carrying the GUS ICR assay and expressing *RuvC*

Cross	No. of stained seedlings	No. of blue sectors*
F_1 (GU-US X <i>RuvC</i>)		
Cross no. 71	337	11
Cross no. 72	30	1
Cross no. 73	211	6
Cross no. 74	250	6
Total	828	24
F_1 (GU-US X Samsun)	402	1

*Blue sectors were monitored in cotyledons and in the first or second true leaf. Most sectors were from cotyledons.

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an 11.4-fold increase in the frequency of blue sectors. Note that in RuvC X GU-US, the data presented in Table 1 is from four crosses (nos. 71–74), each with an independent RuvC transformant, and no obvious differences in ICR frequency was found between the crosses. One difference was in the size of the sectors. Cross no. 74 had small sectors (1–2 cells large), whereas the other crosses had larger sectors (1–16 cells), indicating late recombination in cross no. 74. Tobacco cotyledons can be used to determine the ICR frequency because the development (number of cells and patterns of divisions) of this organ is well understood (29). Based on the frequency and size of blue sectors in cotyledons (data not shown), we estimate that ICR occurs in the 10^{-6} range/genome in the presence of RuvC and in the 10^{-7} range in WT tobacco.

Increased Somatic Crossover in RuvC-Expressing Plants. The effect of RuvC on somatic crossover was determined by using the frequency of twin sectors in T1 *Su/su* Xanthi transgenic plants expressing RuvC transcript (Fig. 1). The sulfur gene (*Su*) controls chlorophyll pigmentation in tobacco. It is characterized by a pale green color in leaves and shoots of heterozygote *Su/su* plants (30). Self-pollination of *Su/su* plants gives rise to progeny that segregate for 1/4 dark green *Su/Su* plants, 1/2 pale green *Su/su* plants, and 1/4 yellow *su/su*

plants that die soon after germination. *Su/su* plants occasionally give rise to simple dark green or white sectors or to twinned (contiguous dark green and white) sectors (Fig. 3A). These sectors can occur as a result of various genetic events, such as point mutations at the sulfur locus, chromosome loss or breakage, chromosomal nondisjunction, or somatic crossover. Carlson (17) has provided direct evidence that most twinned sectors (12 of 13 sectors regenerated and cytologically analyzed) in *Su/su* tobacco plants are derived from somatic crossover (17). We found, via cytological analysis and pollen viability, that transgenic lines carrying RuvC did not show any evidence for chromosomal instability (data not shown). These data, in accordance with Carlson's previous analysis, suggest that twin sectors correspond to somatic crossover events rather than to chromosome loss associated with chromosome gain in the sister cell. The frequency of twinned sectors (average number per leaf) in RuvC-expressing plants and in the WT cultivar used for the production of the transgenic RuvC plants is shown in Fig. 3B. There is a 12-fold higher frequency of twin sectors in RuvC compared with WT plants, suggesting that somatic crossover is significantly increased as a result of RuvC expression. This effect on twin sectors was found in six different transgenic lines expressing the RuvC transcript, ruling out the possibilities of position effect, spontaneous mutation, or somaclonal variation. Moreover we have made several transgenic plants in the *Su/su* background with constructs that do not express RuvC, and these plants did not show increased somatic crossover. Therefore, the mere fact of being transgenic (i.e., to undergo transformation and regeneration and to express antibiotic resistance genes) does not normally increase the rate of somatic crossover.

DISCUSSION

This study shows that overexpression of RuvC is associated with increased HR in higher eukaryotes. This effect was observed for ICR with the somatic crossing-over twin-sector assay (12-fold increase), ICR with the GU-US assay (11-fold), and extrachromosomal plasmid sequences with the ECR bombardment assay (56-fold increase) in tobacco plants.

The stimulating effect of RuvC on HR in plants is surprising as this protein is active at the final steps of the HR process. This effect might result from one or from a combination of the following possibilities. The increased HR frequency might result from a direct RuvC effect: plant cells might contain several heteroduplex DNA molecules, which for some unclear reasons are not efficiently resolved under normal levels of expression. In the WT, cells with unresolved heteroduplexes would stop dividing. Overproduction of RuvC could lead to RuvC-mediated cleavage of these unresolved structures and thus enable cell division to proceed and subsequent twin sector formation. Similarly, in the ECR assay, RuvC expression might enable the resolution of heteroduplexes between the transformed plasmids, which, otherwise (in the WT) would accumulate. It has been shown for mitochondrial DNA that the number of unresolved junctions increases significantly in *cce1* (CCE1 is the yeast homolog to RuvC) yeast mutants (31). Another possible explanation for the RuvC effect is that in plants, Holliday junctions might resolve in favor of the parental configuration (with subsequent gene conversion) to avoid chromosome translocations between repeats. Maybe RuvC removes this bias and permits resolution to give crossover recombinants. RuvC overproduction also might have a non-specific, yet nonetheless direct, effect on HR through non-specific DNA binding, nuclease activity, and subsequent induction of the endogenous HR machinery, in a genome-wide fashion, to repair the damage. It has been found, *in vitro*, that in addition to the high affinity binding capacity to four-way DNA junctions, CCE1 is able to bind duplex DNA molecules, albeit with lower affinity (5).

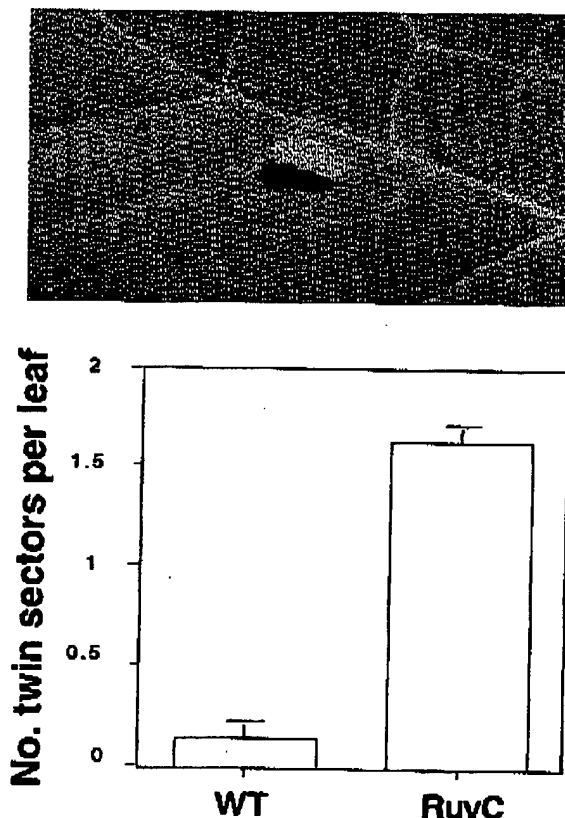


FIG. 3. Frequency of twin sectors in RuvC-expressing *Su/su* plants. A picture of a twin sector is shown on the background of a light green (*Su/su*) tobacco leaf (Upper). The dark green and the yellow sectors are contiguous and probably were formed as a result of somatic crossover (17). The number of twin sectors was counted in 20 leaves per plant. (Lower) The average number of twin spots per leaf is shown with error bars representing the SEM. This average was calculated out of six T1 transgenic plants originating from independent transformation events of *Su/su* plants with RuvC (i.e., $6 \times 20 = 120$ leaves) and three *Su/su* nontransformed plants (i.e., $3 \times 20 = 60$ leaves).

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The effect of RuvC on HR might be specific but indirect. It was discussed earlier that branch migration and resolution of the Holliday junctions may be coupled by direct interactions between RuvA, RuvB, and RuvC proteins, possibly by the formation of a RuvABC complex (4). If so, it is conceivable that the overexpressed RuvC protein recruits RuvA- and RuvB-like plant proteins in the cells to form the complex indispensable for branch migration and resolution of Holliday junctions. Thus, the increased HR frequency might result from increased helicase activity of RuvA and RuvB and may affect earlier steps of HR rather than the final resolution step. So far, plant homologs of RuvC, RuvA, and RuvB proteins have not been identified.

In conclusion, although we do not understand the mechanism by which RuvC stimulates both genomic and extrachromosomal HR in plants, the expression of this protein can lead to the engineering of recombinogenic plants. Such plants, in combination with the development of improved gene targeting vectors, may enable high frequencies of exogenous DNA integration into chromosomal targets via HR and thus facilitate reverse genetics in plants by gene targeting.

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